Caenorhabditis elegans CED-4 stimulates CED-3 processing and CED-3-induced apoptosis

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Background: Programmed cell death or apoptosis is a key feature of normal development, tissue homeostasis and disease progression in metazoans. Genetic studies in the nematode *C. elegans* have identified three key genes involved in apoptosis, *ced-3*, *ced-4* and *ced-9*. Expression of *ced-3* and *ced-4* is required for the induction of cell death, whereas expression of *ced-9* is necessary to inhibit cell death. The precise mechanism by which these genes influence the life or death decision of a cell is not known. In this study, we have expressed the genes in an insect cell line to explore their role in the apoptotic pathway.

Results: Co-expression of *ced-4* with *ced-3* in insect cells stimulated both the induction and the level of CED-3-mediated apoptosis. Stimulation of CED-3-dependent apoptosis by CED-4 was accompanied by accelerated processing of CED-3, which was dependent on the presence of a wild-type CED-3 pro-domain and a conserved lysine residue within a putative ATP/GTP-binding motif of CED-4. Co-expression of *ced-9* with *ced-4* and *ced-3* inhibited the ability of CED-4 to stimulate CED-3 processing and CED-3-dependent apoptosis. Although a temperature-sensitive CED-9 mutant was unable to block CED-4 activity and failed to associate with CED-4, a deletion mutant of CED-9 lacking the carboxy-terminal hydrophobic domain could associate with CED-4 and block CED-4 activity.

Conclusions: Our results establish a role for CED-4 in the processing of CED-3 and the stimulation of CED-3-induced apoptosis. Furthermore, we show that CED-9 achieves its anti-apoptotic effect by associating with CED-4 and blocking the ability of CED-4 to process CED-3.

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Background

Apoptosis is an evolutionarily conserved process that plays a major role in development, tissue homeostasis and disease processes in metazoans [1–3]. Deregulated cell death can lead to abnormal development, degenerative diseases and cancer progression. Several genes that regulate the apoptotic pathway have been identified in both vertebrates and invertebrates; for example, genetic studies in *Caenorhabditis elegans* have revealed that 14 genes are involved in the 131 somatic cell deaths that occur during normal nematode development [4]. Of these 14 genes, *ced-3* and *ced-4* are both required for programmed cell death to occur, and *ced-9* is required to prevent apoptosis from occurring in cells that normally survive [5–7]. Despite CED-3 — a cysteine-dependent aspartate-specific protease, or caspase — and CED-9 — a homolog of the mammalian anti-apoptotic protein Bcl-2 — having mammalian counterparts, CED-4 shows no significant homology to any reported mammalian sequence. Although both CED-3 and CED-4 are required for all somatic cell deaths occurring during development of the worm, ectopic expression of CED-3 alone, in the absence of CED-4, can induce apoptosis in *C. elegans* [8]. In contrast, effective induction of apoptosis by CED-4 requires functional CED-3, and the effect of overexpression of CED-4 is mitigated by overexpression of CED-9 [8]. These results suggest that CED-4 may act upstream of CED-3 or in parallel to CED-3 in the apoptotic pathway, although the precise hierarchical order of the three genes in the pathway is still not clear. A physical association of CED-4 with both CED-3 and CED-9 has recently been reported, however [9–12]. As all known caspase family members are synthesized as proenzymes and are known to be activated by processing during induction of apoptosis, it is possible that CED-4 influences the activation of CED-3, and that CED-9 may block this regulatory function of CED-4.

We have tested this hypothesis by transiently expressing these three genes in Sf-21 cells, an insect cell line that requires no additional protein or RNA synthesis for the induction of apoptosis and responds to a variety of apoptotic stimuli [13–15]. In this study, we have found that CED-4 accelerated the processing of CED-3 and increased

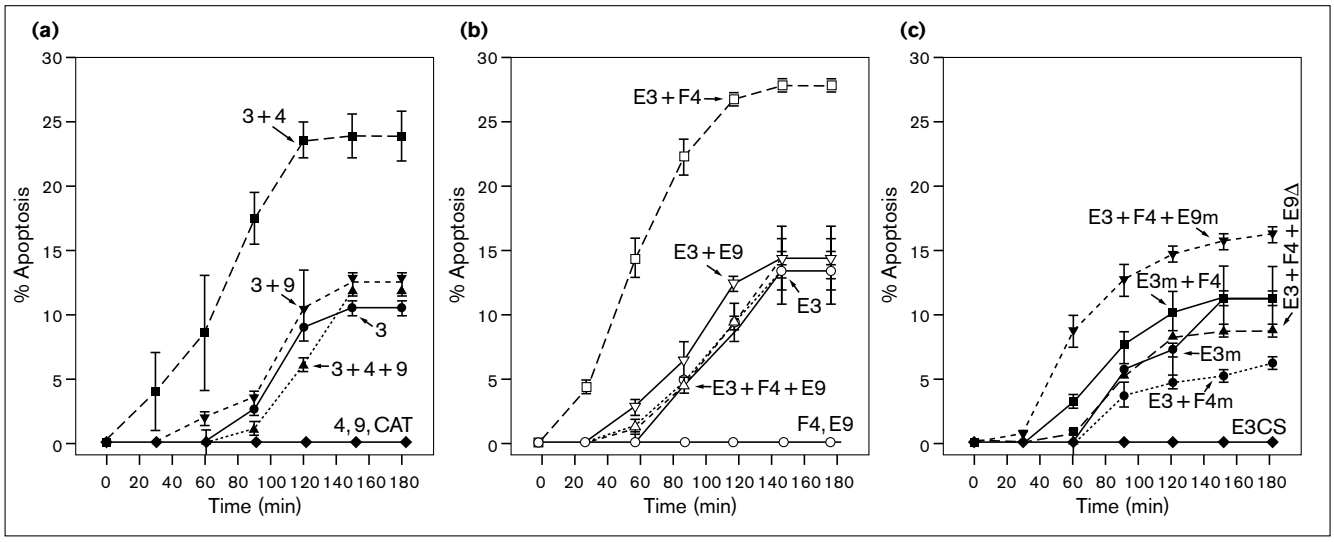
the level of CED-3-induced apoptosis, when co-expressed with CED-3 in Sf-21 cells. Furthermore, the CED-4-mediated processing of CED-3, and subsequent increase in apoptosis, were dependent on the presence of a wild-type pro-domain in CED-3. The conserved lysine residue in the putative ATP/GTP-binding site motif (p-loop) of CED-4 was important for CED-4 activity. Consistent with the anti-apoptotic role of CED-9, both the wild-type protein and a carboxy-terminal deletion mutant form lacking the hydrophobic domain inhibited CED-4 activity and associated with CED-4. Our results establish a role for CED-4 in stimulating CED-3 processing and CED-3-induced apoptosis, and demonstrate that CED-9 inhibits CED-4 activity by direct interaction.

Results and discussion

CED-4 accelerates and increases CED-3-induced apoptosis

The cDNAs encoding CED-3, CED-4 (the pro-apoptotic form, CED-4_S) and CED-9 were inserted into similar expression plasmids so that their expression was under the control of the *Drosophila hsp70* promoter [13]. This promoter is active in Sf-21 cells and can be induced further by heat-shock treatment [13]. The three genes, *ced-3*, *ced-4* and *ced-9*, were expressed transiently either alone or in combination in Sf-21 cells and the kinetics of apoptosis induction were studied. Cells in randomly selected fields were repeatedly photographed over a period of 3 hours after heat shock and the number of apoptotic cells, identified by the characteristic blebbing morphology, was used as a measure of the level of apoptosis. Transient expression of *ced-3* in Sf-21 cells induced apoptosis around 60–90 minutes after induction by heat shock. By 120 minutes after induction, approximately 9% of the cells were apoptotic (Figure 1a). Although the expression of *ced-4* alone did not induce apoptosis, co-expression of *ced-4* with *ced-3* induced apoptosis by 30 min after induction, and resulted in a 2–3-fold increase in the total number of cells undergoing apoptosis by 120 minutes (Figure 1a). Expression of *ced-3* or co-expression of *ced-3* with *ced-4* in Sf-21 cells induced typical features of apoptosis, including membrane blebbing, apoptotic-body formation and oligonucleosomal-ladder formation (data not shown). Co-expression of *ced-9* with *ced-4* and *ced-3* delayed the appearance of apoptotic cells and decreased the total level of apoptosis to approximately that observed following expression of *ced-3* alone (Figure 1a). Expression of *ced-9*

Figure 1



Kinetics of apoptosis induced by CED-3 with or without co-expression of CED-4. Sf-21 cells (5×10^5 per 35 mm tissue culture dish) were transfected with combinations of plasmids expressing untagged or epitope-tagged forms of the proteins. A plasmid expressing chloramphenicol acetyl transferase (CAT) was used as a negative control and to balance plasmid DNA concentrations so that a total amount of 0.25 μg of plasmid DNA was present in each transfection. Cells were heat-shocked for 30 min at 42°C, 6 h after transfection. To assess the level of apoptosis, the same field of cells was repeatedly photographed using an Olympus photomicrographic system attached to an Olympus IX50 microscope. Pictures were taken at 30 min intervals beginning at time zero, which represents the end of the heat-shock treatment. The number of apoptotic cells (identified by blebbing and apoptotic-body formation [13,15]) per field of a hundred cells was calculated and data from at least two independent fields were used to calculate the percentage apoptosis. **(a)** Sf-21 cells were transfected with plasmids expressing CED-3 (3), CED-3 + CED-4 (3 + 4), CED-3 + CED-4 + CED-9 (3 + 4 + 9), CED-3 + CED-9 (3 + 9), CED-4 (4), CED-9 (9) and CAT (CAT). **(b)** Sf-21 cells were transfected with plasmids expressing EpiCED-3 (E3), EpiCED-3 + FlagCED-4 (E3 + F4), EpiCED-3 + FlagCED-4 + EpiCED-9 (E3 + F4 + E9), EpiCED-3 + EpiCED-9 (E3 + E9), FlagCED-4 (F4), and EpiCED-9 (E9). **(c)** Sf-21 cells were transfected with plasmids expressing EpiCED-3m (E3m), EpiCED-3m + FlagCED-4 (E3m + F4), EpiCED-3 + FlagCED-4m (E3 + F4m), EpiCED-3+ FlagCED-4 + EpiCED-9Δ (E3 + F4 + E9Δ), EpiCED-3 + FlagCED-4 + EpiCED-9m (E3 + F4 + E9m) and EpiCED-3CS (E3CS).

alone did not induce apoptosis and, when co-expressed with *ced-3*, *ced-9* had essentially no effect on the timing or level of CED-3-induced apoptosis (Figure 1a). These results suggest that CED-9 blocks the ability of CED-4 to stimulate CED-3-induced apoptosis.

In preparation for further studies, we added epitope tags to CED-3, CED-4 and CED-9 and checked that these tagged proteins were functionally similar to the wild-type proteins. The presence of carboxy-terminal HA.11 and His_6 tags in EpiCED-3, amino-terminal HA.11 and His_6 tags in EpiCED-9, and amino-terminal Flag and His_6 tags in FlagCED-4 did not significantly alter the function of these proteins (Figure 1b). Like wild-type CED-4, FlagCED-4 induced apoptosis by 30 minutes after heat-shock induction and increased the total number of apoptotic cells when co-expressed with EpiCED-3 (Figure 1b), but did not induce apoptosis when expressed alone (Figure 1b). Likewise, EpiCED-9 had essentially no effect on EpiCED-3-induced apoptosis, but blocked FlagCED-4-mediated stimulation of EpiCED-3-dependent apoptosis (Figure 1b).

Mutational analysis of CED-3, CED-4 and CED-9

To further understand the interactions between CED-3, CED-4 and CED-9, we introduced mutations into the tagged versions of the three proteins and studied the effects of the mutant proteins on apoptosis (Figure 1c). Expression of each mutant was confirmed by western analysis (data not shown, see below). EpiCED-3CS, a CED-3 mutant with a serine substitution for the essential cysteine at its catalytic site, failed to induce apoptosis. EpiCED-3m, containing a previously described null mutation that changes the leucine residue at position 27 within the pro-domain to phenylalanine [6], induced apoptosis with similar kinetics to wild-type CED-3 (compare Figure 1c to Figure 1a,b). This indicates that the mutation in the pro-domain did not affect the basal level of apoptosis that is normally observed upon expression of wild-type CED-3 alone (Figure 1a,b). Co-expression of EpiCED-3m with FlagCED-4 failed to accelerate the appearance of apoptotic cells, however, and only 11% of the cells were apoptotic 120 minutes after induction, compared with the stimulation and increased levels (28%) observed upon co-expression of wild-type EpiCED-3 and FlagCED-4 (compare Figure 1c with Figure 1b).

These data indicate that the pro-apoptotic effect of CED-4 is mediated through CED-3, and that the pro-domain of CED-3 is important for the regulation of CED-3 by CED-4. A CED-4 mutant containing a mutation in the putative ATP/GTP-binding motif of FlagCED-4, FlagCED-4m, failed to stimulate CED-3-dependent apoptosis (Figure 1c) and reduced the total amount of apoptotic cells resulting from expression of EpiCED-3 alone (Figure 1b). This result indicates that the sequence of the putative ATP/GTP-binding motif is important for CED-4 function, and is consistent with the inability of a similar CED-4 mutant to induce cell death in *Schizosaccharomyces pombe* [12].

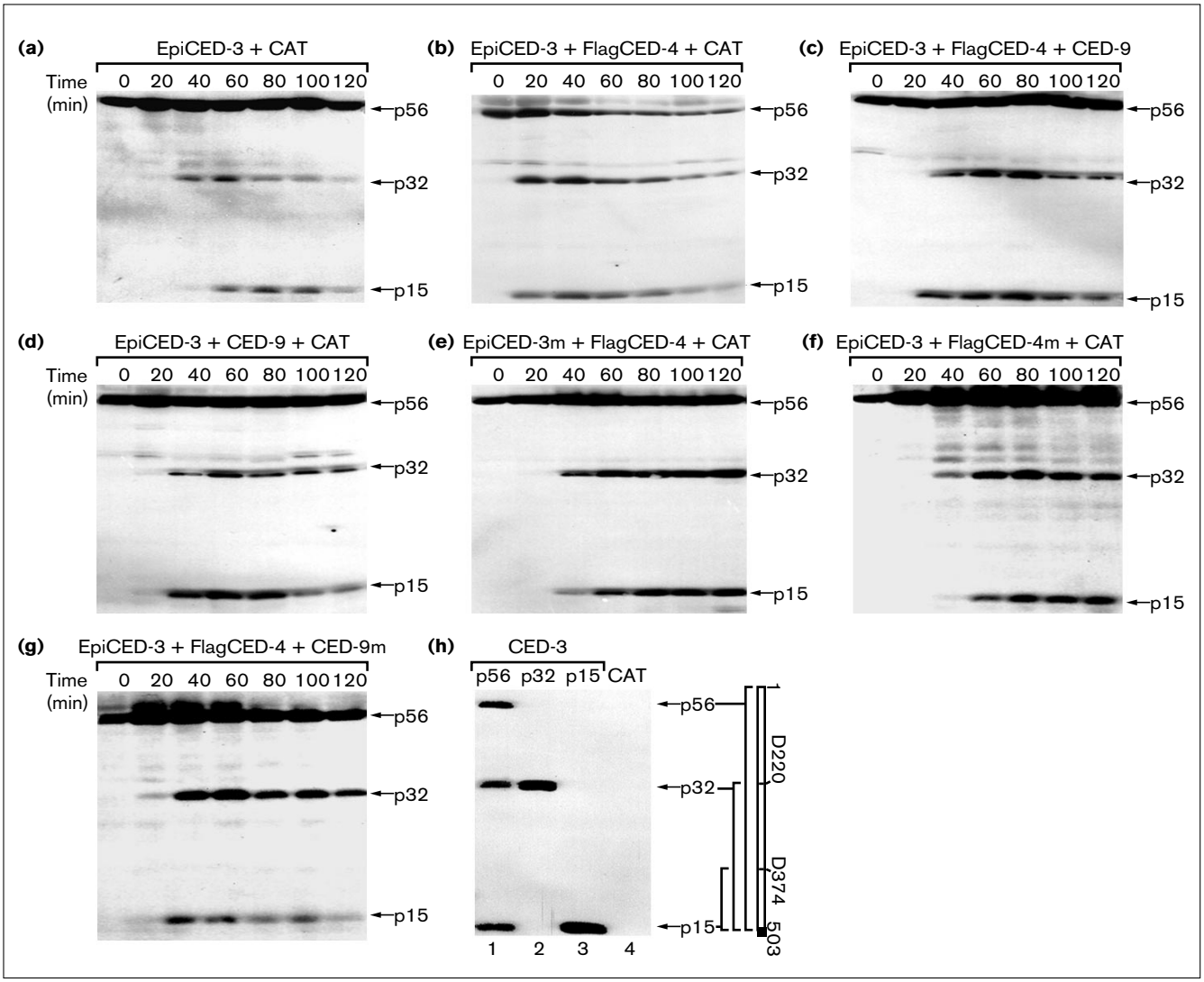
When co-expressed with FlagCED-4 and EpiCED-3, EpiCED-9m, a mutant of CED-9 (n1653ts) that is temperature sensitive in *C. elegans* [7], was only partially effective in blocking FlagCED-4 function in Sf-21 cells compared with the wild-type EpiCED-9 (compare Figure 1c with Figure 1b). Human Bcl-2 and other members of the Bcl-2 family contain a conserved hydrophobic tail at their carboxyl terminus [16], deletion of which has minimal effects on the ability of Bcl-2 to block apoptosis in cell culture [17]. To test the effect of deleting the hydrophobic tail of CED-9, we constructed a deletion mutant of CED-9, EpiCED-9Δ, lacking the carboxy-terminal 33 amino acids. When co-expressed with FlagCED-4 and EpiCED-3, EpiCED-9Δ blocked the activity of FlagCED-4 as effectively as the wild type EpiCED-9 (compare Figure 1c to Figure 1b).

CED-4-mediated acceleration of CED-3 processing is blocked by CED-9

To further investigate the effect of CED-4 on CED-3-induced apoptosis, the kinetics of EpiCED-3 processing in the presence and absence of CED-4 were studied. We expressed EpiCED-3 transiently with other proteins, as indicated in Figure 2, and studied CED-3 processing [18] by immunoblot analysis. Expression of EpiCED-3 resulted in the processing of the 56 kDa precursor form to the tagged intermediate p32 and the mature p15 subunit around 40 minutes after induction of expression (Figure 2a,h); as the epitope tag in EpiCED-3 is at the carboxyl terminus, the other processed subunit of the mature caspase, p17, could not be observed in this analysis. When co-expressed with FlagCED-4, the processed forms of EpiCED-3 were observed as early as 20 minutes after induction (Figure 2b). This finding indicates that CED-4 functions to stimulate CED-3 processing and is consistent with the acceleration of induction of apoptosis following co-expression of CED-3 and CED-4 (Figure 1a). No processing was observed for EpiCED-3CS in the presence or absence of CED-4, because only the 56 kDa mature form of EpiCED-3CS was observed 60 minutes after heat-shock induction (data not shown), suggesting that CED-4 stimulates the autocatalytic processing of CED-3.

Although co-expression of CED-9 and EpiCED-3 did not alter the kinetics of EpiCED-3 processing (Figure 2d), co-expression of CED-9 with FlagCED-4 and EpiCED-3 blocked the acceleration of EpiCED-3 processing by FlagCED-4 (Figure 2c). This inhibition provides direct evidence that CED-9 blocks CED-4 function and is consistent with our observation that CED-9 blocks the stimulation of CED-3-induced apoptosis by CED-4.

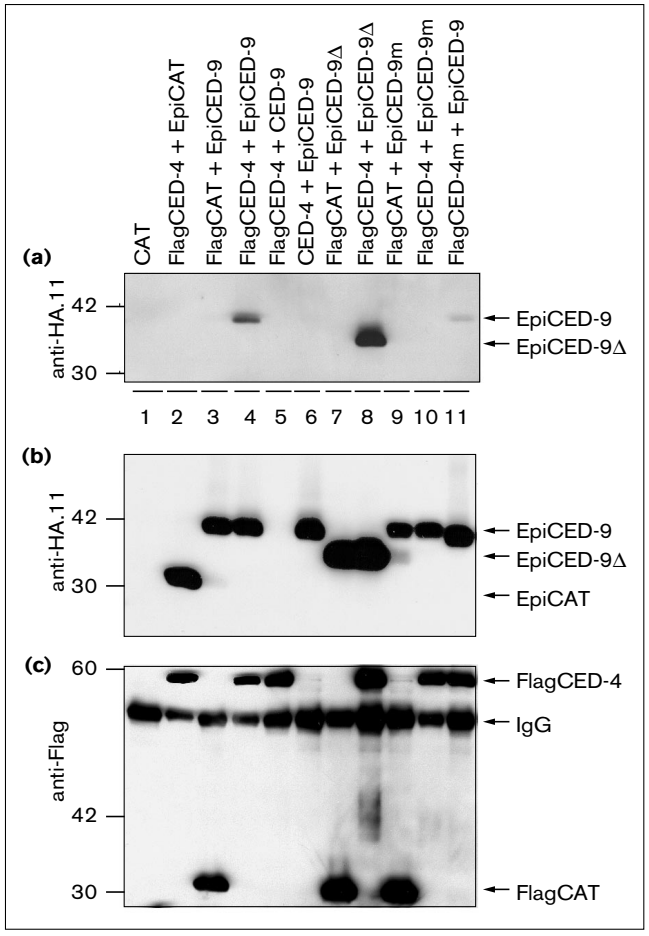
Figure 2



CED-4 accelerates CED-3 processing and CED-9 blocks the effect of CED-4 on CED-3 processing. **(a–h)** Sf-21 cells (5×10^5 per 35 mm dish) were transfected with the appropriate expression plasmids as indicated above each panel. Cells were heat-shocked at 42°C for 30 min, 6 h after transfection. Samples were then collected at 20 min intervals beginning at time zero which represents the end of the heat-shock treatment. Immunoblot analysis of HA.11-tagged CED-3 using anti-HA.11 monoclonal antibody was performed. Longer exposures are shown of (f,g) than of the other panels in order to detect any processed CED-3 products 20 min after induction. Shorter exposures of (g) showed that the level of the p56 form of CED-3 decreased in a manner similar to that seen in (b). (h) Cells transfected with plasmids expressing full-length EpiCED-3 (p56) (lane 1), the p32 form of EpiCED-3 (lane 2), the p15 subunit of EpiCED-3(lane 3) and the control CAT (lane 4) were collected 60 min after heat-shock induction. The full-length EpiCED-3 in Sf-21 cells was processed into fragments corresponding to the epitope-tagged p32 and p15 forms (lane 1), as revealed by the co-migration of these fragments with the products derived independently from plasmids expressing the epitope-tagged p32 (lane 2) and p15 (lane 3) forms. A schematic representation of the full-length CED-3 (p56) protease, with the carboxy-terminal HA.11 tag (represented as a solid box at the carboxyl terminus) and the processed products, is shown on the right.

EpiCED-3m, containing a mutation in the pro-domain of CED-3 [5], showed similar kinetics of processing as wild-type CED-3. Unlike wild-type CED-3, however, the processing of EpiCED-3m was not stimulated by co-expression with FlagCED-4 (Figure 2e), establishing the importance of the CED-3 pro-domain for the CED-4-mediated effect on CED-3 processing. The CED-4 mutant containing a mutation in the putative ATP/GTP-binding motif, FlagCED-4m, did not stimulate EpiCED-3 processing, confirming the importance of this motif for CED-4 function (Figure 2f). When co-expressed with FlagCED-4 and EpiCED-3, the temperature-sensitive CED-9 mutant, CED-9m, only partially blocked the appearance of the processed forms of CED-3 20 minutes after induction (Figure 2g), confirming that this mutant is partially defective in Sf-21 cells.

Figure 3



CED-4 associates with CED-9 and CED-9Δ but not with CED-9m. **(a)** Sf-21 cells (5×10^5) were transfected with plasmids expressing genes encoding the indicated FLAG-tagged and HA-tagged proteins under the control of the *hsp70* promoter. Cells were heat-shocked 16 h after transfection and cell lysates were prepared 4 h after heat-shock induction. FLAG-tagged proteins were immunoprecipitated using anti-FLAG monoclonal antibody resin and the co-precipitating HA-tagged proteins were then detected by immunoblot analysis using an anti-HA.11 polyclonal antibody. **(b)** The expression of the HA-tagged proteins in the lysates used in (a) was confirmed by immunoblot analysis using anti-HA.11 monoclonal antibody. **(c)** The membrane in (a) was reprobed with anti-FLAG monoclonal antibody to confirm the expression of the FLAG-tagged proteins. The size standards in kDa are shown on the left of the panels.

CED-9 associates with CED-4 in Sf-21 cells

As CED-9 blocked CED-4 function but had no effect on CED-3 processing, we examined the possibility that CED-9 blocked CED-4 activity by physical interaction. We tested this possibility by co-expressing FlagCED-4 transiently with wild-type EpiCED-9 or mutants of EpiCED-9. Flag-tagged or HA-tagged CAT (FlagCAT and EpiCAT, respectively) were used as controls. Consistent with recent reports [9–12], we found that both EpiCED-9 and the active EpiCED-9Δ mutant lacking the hydrophobic tail specifically co-precipitated with FlagCED-4 (Figure 3a, lanes 4,8), but not with FlagCAT. Thus, both CED-9 and EpiCED-9Δ can associate specifically with CED-4 in insect cells, correlating with the ability of both of these proteins to block CED-4 activity. In contrast with the fully active forms of CED-9, the partially defective mutant of CED-9, EpiCED-9m, did not co-immunoprecipitate with FlagCED-4 under the same conditions, even though it was expressed efficiently (Figure 3a, lane 10). In the case of the functionally inactive CED-4 mutant, FlagCED-4m, we detected only a low level of co-precipitating EpiCED-9 (Figure 3a, lane 11), despite efficient expression of both the proteins (Figure 3b,c, lane 11), indicating a weak interaction of this mutant form of CED-4 with CED-9. The absence of any interaction between FlagCED-4 and EpiCAT also confirmed the specificity of the CED-4–CED-9 interaction. These results are consistent with the recently reported interaction between CED-9 and CED-4 [9–12] and provide a mechanism by which CED-9 blocks CED-4 activity.

Conclusions

Our results indicate that the role of CED-4 in programmed cell death is to stimulate CED-3 processing, thereby generating an active caspase, and that the activity of CED-4 is regulated by its interaction with CED-9. In *C. elegans*, the requirement for CED-4 in programmed cell death during development can be bypassed by overexpression of CED-3 [8]. This suggests that, in normal developing *C. elegans* embryos, the level of processed, active CED-3 is below the critical threshold required for induction of apoptosis. Our results suggest that, under normal physiological conditions, CED-4 stimulates CED-3 processing in a CED-9-regulated manner. This hypothesis is supported by our analysis of mutant forms of these proteins. The CED-3m pro-domain mutant acts as a null mutant of CED-3 in *C. elegans*, but has the same level of pro-apoptotic activity as wild type CED-3 when expressed alone in Sf-21 cells. However, the inability of this CED-3 mutant to respond to CED-4-mediated processing in Sf-21 cells suggests that CED-3m cannot be activated via its pro-domain, accounting for the null phenotype of this mutant in *C. elegans*. As CED-4 also associates with the pro-domain of CED-3 [10], CED-4-mediated activation of CED-3 is likely to be achieved, at least in part, by an interaction between CED-4 and the CED-3 pro-domain. The mutant form of CED-9, CED-9(n1653ts), is impaired in its ability to regulate CED-4 activity and is unable to bind CED-4, correlating with the *in vivo* mutant phenotype of CED-9(n1653ts) observed in *C. elegans*. The association of CED-4 with CED-9 suggests that the stimulation of CED-3 processing by CED-4, and the ultimate fate of the cell, may be regulated by the relative expression levels of CED-4 and CED-9.

Materials and methods

Cells

Spodoptera frugiperda IPLB-SF21(Sf-21) cells [19] were maintained at 27°C in TC-100 medium (Gibco BRL) supplemented with 10% fetal bovine serum and 0.26% tryptose broth. These cells undergo apoptosis in response to different stimuli and do not require fresh protein or RNA synthesis for the induction of apoptosis [13–15].

Plasmid constructs and site-directed mutagenesis

The plasmids used in the kinetic studies were derived by replacing the chloramphenicol acetyl transferase (CAT) gene in a previously described vector phsp70PLVI+CAT [13], thereby placing all genes under the control of the *Drosophila hsp70* promoter. This promoter is active in Sf-21 cells and can be induced further by heat shock [13]. Plasmids phspced-3VI+, phspced-4VI+ and phspced-9VI+ contain sequences encoding CED-3, CED-4 and CED-9, respectively. The epitope-tagged versions of the genes studied were also constructed in phsp70PLVI+ vectors (phsEpiHisVI+, phsFlagHisVI+, and phsC-EpiHisVI+) which provided the appropriate tags as amino-terminal or carboxy-terminal fusions. All the mutants were derived from epitope-tagged expression constructs by site-directed mutagenesis [20]. The mutants contained the following changes: in EpiCED-9Δ, the last 33 amino acids, constituting the putative hydrophobic tail of the wild-type CED-9, were deleted; in EpiCED-3CS, the cysteine residue at position 364 within the active site was replaced by a serine residue; EpiCED-3m, a previously described null mutant in *C. elegans* [5], has a leucine to phenylalanine replacement at position 27 within the pro-domain; in FlagCED-4m, the lysine residue at position 165 within a putative ATP/GTP-binding motif was replaced by a methionine; and in EpiCED-9m, a previously reported temperature-sensitive null mutant of CED-9 [16], the tyrosine residue at position 149 was replaced with asparagine. CED-9m, an untagged version of EpiCED-9m, was derived from EpiCED-9m by subcloning the coding region of CED-9m into phsp70PLVI+CAT. Plasmids expressing the HA.11-tagged p32 (amino acids 221–503) and the p15 (amino acids 375–503) forms of CED-3 were derived by cloning the appropriate PCR products into phsC-EpiHisVI+.

Western blot analysis

Proteins were resolved by SDS–PAGE on 12% acrylamide gels and transferred onto PVDF membranes (Millipore), blocked and incubated with anti-HA.11 mouse IgG (BABCO), followed by anti-mouse horseradish peroxidase-conjugated IgG (Promega). Immunoblots were developed by enhanced chemiluminescence (Amersham).

Immunoprecipitation

Cells were collected 4 h after heat shock and lysed in NP-40 lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1.0% Nonidet P-40, 1 mM DTT) containing protease inhibitor cocktail (Pharmingen). Proteins in the soluble fraction of the lysate were precipitated using anti-FLAG M2 affinity resin (Eastman Kodak Company), extensively washed in NP-40 lysis buffer, and resolved by SDS–PAGE on a 10% acrylamide gel. Separated proteins were electophoretically transferred to PVDF membranes and probed with polyclonal HA.11 anti-rabbit IgG to detect co-precipitating HA.11 epitope-tagged proteins. Expression of HA.11-tagged or Flag-tagged proteins in the detergent soluble fraction was determined by western analysis using either anti-HA.11 mouse monoclonal IgG or anti-Flag mouse monoclonal IgG and an appropriate secondary antibody conjugated to horseradish peroxidase.

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